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RESEARCH PAPER

Co-utilization of Maltose and Sodium Acetate via Engineered *Corynebacterium glutamicum* for Improved Itaconic Acid Production

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Abstract For growth and energy, *Corynebacterium glutamicum* has the ability to assimilate numerous carbon sources in the form of either single or combined substrates. During the growth of *C. glutamicum* on substrate mixtures, it has shown the ability to co-metabolize the majority of these carbon sources and displays monophasic growth, unlike other microorganisms such as *Escherichia coli* and *Bacillus subtilis*, which exhibit either diauxic or biphasic growth. Here, a recombinant strain of *C. glutamicum* ATCC 13032 was selected for the use in the production of itaconic acid (IA), which is a promising biochemical building block that could be an alternative material for polymer synthesis. For this purpose, an engineered *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was constructed by introducing the plasmid pCH-$cadA_{opt}$, which expressed a *cis*-aconitate dehydrogenase gene (*cadA*) that originated from *Aspergillus terreus*. The production of IA was evaluated using a combined mixture of maltose and sodium acetate. The monophasic growth of *C. glutamicum* in the presence of maltose and sodium acetate was observed and showed final IA titer of 12.63 g/L, and a molar yield of 0.38 mol/mol after 240 h of cultivation. The present study suggests the possibility of utilizing a mixture of carbon sources by *C. glutamicum* to improve IA production.

Keywords: itaconic acid, *Corynebacterium glutamicum*, sodium acetate, monophasic growth, flux balance analysis

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1. Introduction

A wide range of carbon and energy sources can be utilized by many microorganisms, and they adapt their metabolism and enzyme systems to the presence of a given carbon source or mixture of substrates. This adaptive process depends mainly on the substrate or on the repression of the genes of catabolism [1]. Many organisms predominantly uptake only one substrate, and begin to utilize other available substrate(s) after the initial carbon source is completely consumed [2]. This phenomenon is known as either biphasic growth or diauxic growth where the preferred substrate generally assists in a better growth rate. Optimal growth is a result of repression of the expression genes that are responsible for the enzymatic catabolism of a subordinate substrate as a result of the availability of the catabolites that result from the preferred carbon source, and this is referred to as carbon catabolite repression [1,3,4]. Otherwise, a number of microorganisms simultaneously utilize more than one carbon source without possessing any catabolite repression in the case of the availability of diverse substrates [5,6]. *Corynebacterium glutamicum* is a gram-positive, non-sporulating and non-endotoxin bacteria that is considered a safe host [7], which was initially recognized for its ability to produce many amino acids, and it is now widely used in the production of organic acids, diamines or alcohols [8]. *C. glutamicum* also has the ability to harness various carbon sources to support the best growth and supply energy from

sugars such as glucose, sucrose, fructose, mannose, maltose, and ribose.

Additionally, *C. glutamicum* has shown the ability to grow on either one carbon source or on a mixture of substrates [9]. Microorganisms like *Escherichia coli* and *Bacillus subtilis* display diauxic or biphasic growth. The growth of *C. glutamicum* on substrate mixtures, however, demonstrates the ability to co-metabolize many carbon sources and display monophasic growth as shown in the mixtures of glucose and lactate, glucose and pyruvate, glucose and sodium acetate, and glucose and propionate [10-15]. Furthermore, the impact of maltose on sodium acetate and glucose consumption has been investigated for its role in enhancing the production of L-valine via *C. glutamicum* [9].

Itaconic acid (IA) is regarded as a promising bio-based product that could be used in polymer production as an alternative to acrylic and methacrylic fibers. Furthermore, it can replace many chemical intermediates such as styrene, 2-methyl-1,4-butanediol, and 3-methyl tetrahydrofuran [16-18].

However, many microorganisms are involved in the production of IA, the highest concentration of IA (160 g/L) reached by using the natural producer filamentous fungus *Aspergillus terreus*, and now 400 t of IA are produced every year using glucose [19]. There are many obstacles in the production of IA by *A. terreus*. These include the high cost of production, sensitivity to shear stress, and changes in the oxygen supply where production can be stopped for many hours even if oxygen levels are depleted for a short duration [20]. Moreover, *A. terreus* is considered a human pathogen that belongs to risk group 2 [21].

Consequently, recently many studies have focused on other possible microorganisms other than *A. terreus* for IA production. These alternatives have included fungi such as *Ustilago maydis* [22-24] and bacteria such as *E. coli* [25-27] and *C. glutamicum* [28,29]. Thus far, *E. coli* has achieved the best IA titer among bacteria at final IA titer of 46.9 g/L using a temperature-controlled production strain [26]. The highest IA concentration reached by *C. glutamicum* was 29.2 g/L by using sodium acetate as a sole carbon source via pH-controlled fed-batch fermentation in 42 L bioreactor in a process that combined pH and DO-coupled feeding of acetate [28].

In this study, the effect of different carbon sources on IA production in *C. glutamicum* was investigated. A mixture of substrates was also used to evaluate their effect on cell growth and IA production via *C. glutamicum*. The heterologous expression of a codon-optimized, *cis*-aconitate decarboxylase gene from *A. terreus* in the wild-type strain *C. glutamicum* ATCC 13032 resulted in a low IA production titer by using numerous carbon sources such as glucose, fructose, sucrose, maltose and mannose. Sodium acetate, however, was the carbon source that led to the highest IA production titer, while maltose led to optimal cell growth. Higher concentrations of sodium acetate, however, led to a decrease in cell growth and a longer lag phase. Consequently, the combination of sodium acetate and maltose led to improved cell growth and IA production by the engineered strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. The utilization of maltose and sodium acetate as the main carbon sources, resulted in an increase in IA production to a final titer of 12.63 g/L IA. This reflected the possibility of enhancing the cell growth and IA production in *C. glutamicum* by using mixture of carbon sources.

2. Materials and Methods

2.1. Recombinant DNA work

The synthesis of all oligonucleotides used in this study was accomplished by Life Technologies Japan Ltd. Polymerase chain reaction (PCR) was carried out by following the standard protocols [30] with KOD one Start polymerase (Toyobo). All the restriction enzymes used in the cloning were obtained from New England Biolabs. Ligation was conducted using a Ligation high Kit (Toyobo) according to the manufacturer's instructions. Plasmid DNA was isolated using a DNA extraction kit (Cosmo Genetech). Transformation of *C. glutamicum* was performed by electroporation as previously described [31]. The constructed plasmids were checked by colony PCR, then were confirmed via DNA sequencing using the 3500 Genetic Analyzer (Applied Biosystems). DNA sequence data were then analyzed using the Genetyx program (Software Development).

2.2. Construction of bacterial strains and plasmids

In this study, all bacterial strains and plasmids used or constructed are summarized in Table 1. BHIS medium, which is a mixture of 37 g/L of brain heart infusion (BHI) medium (Difco Laboratories) and 91 g/L sorbitol, was used for *C. glutamicum*, and Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used for *E. coli*. The cultivation of *E. coli* strains was performed routinely by growing at 37ºC for 16 h in LB medium containing 15 g/L agar and supplemented with kanamycin (50 μg/mL). *C. glutamicum* strains were cultured on 37 g/L BHI media with 15 g/L agar, supplemented with kanamycin (25 μg/mL), and incubated at 30ºC for 24–48 h.

To construct the expression plasmids pCH-cad_{opt}, pCH-$cad_{opt}acnB_{opt}$, and pCH-$acnB_{opt}cad_{opt}$, the *cadA* gene from *A. terreus* with a codon-optimized sequence for *C. glutamicum,* and the fusion proteins $cadA_{opt}acnB_{opt}$ and $acnB_{opt}cadA_{opt}$ with the *cadA* gene from *A. terreus* fused with the aconitase gene (*acnB*) from *C. glutamicum* by $(GGGGS)_3$

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relative characteristics	Resource/ reference
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1,* Δ (*lac-proAB*)/*F'* [*tra*D36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Takara Bio
E. coli NovaBlue	*endA1, hsdR17* (*rK12*$^-$*mK12*$^+$), *supE44, thi-1 gyrA96*, *relA1, lacrecA1*/*F'* [*proAB*$^+$ *lacI*^{q} *Z1*M15 Tn*10* TetR]*;* used for gene cloning	Novagen
C. glutamicum ATCC 13032	Wild-type, biotin-auxotrophic	ATCC
C. glutamicum ATCC 21799	Wild-type, aminoethylcysteine-resistant, L-leucine auxotroph, lysine-producing strain	ATCC
C. glutamicum ATCC 13287	Wild-type, homoserine auxotrophic	ATCC
C. glutamicum ATCC 21253	Wild-type, homoserine and leucine auxotrophic	ATCC
C. glutamicum ATCC 13032 / pCH-*cadA*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 13032 pCH-*cadA*$_{opt}$*acnB*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus* fused to the synthetic codon-optimized *acnB* gene of *C. glutamicum*	This study
C. glutamicum ATCC 13032 pCH-*acnB*$_{opt}$*cadA*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *acnB* of *C. glutamicum* fused to the synthetic codon optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 21799 / pCH-*cadA*$_{opt}$	ATCC 21799 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 21253 / pCH-*cadA*$_{opt}$	ATCC 21253 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 13287 / pCH-*cadA*$_{opt}$	ATCC 13287 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
pMA-*cadA*$_{opt}$	*AmpR*; *ColE1* origin, containing the synthetic *cadA* gene from *A. terreus*, codon-optimized for *C. glutamicum*	This study
pMK-*acnB*$_{opt}$*cadA*$_{opt}$	*KanR*; *ColE1* origin, containing the synthetic *cadA* gene of *A. terreus* fused to the synthetic *acnB* gene of *C. glutamicum*, codon-optimized for *C. glutamicum*	This study
pMK-*cadA*$_{opt}$*acnB*$_{opt}$	*KanR*; *ColE1* origin, containing the synthetic *cadA* gene of *A. terreus* fused to the *acnB* gene of *C. glutamicum*, codon-optimized for *C. glutamicum*	This study
pCH	*KanR; E. coli-Corynebacterium* sp. shuttle vector	[32]
pCH-*cadA*$_{opt}$	*KanR*; containing the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
pCH-*cadA*$_{opt}$*acnB*$_{opt}$	*KanR*; containing the synthetic codon-optimized *cadA* gene of *A. terreus* fused to the synthetic codon-optimized *acnB* gene of *C. glutamicum*	This study
pCH-*acnB*$_{opt}$*cadA*$_{opt}$	*KanR*; containing the synthetic codon-optimized *cadA* gene of *A. terreus* fused to the synthetic codon-optimized *acnB* of *C. glutamicum*	This study

linker protein were chemically synthesized by GeneScript Biotech and delivered as plasmids pMA-*cadA*$_{opt}$, pMK-*acnB*$_{opt}$*cadA*$_{opt}$, and pMK-*cadA*$_{opt}$*acnB*$_{opt}$. The pCH shuttle vector (*C. glutamicum*-*E. coli* shuttle vector, which is kanamycin resistant and contains a high expression constitutive promoter) [32] was used for expression of *cadA*$_{opt}$ *gene,* and the fusion proteins; *cadA*$_{opt}$*acnB*$_{opt}$ and *acnB*$_{opt}$*cadA*$_{opt}$ in the wild-type strain *C. glutamicum* ATCC 13032. The codon-optimized *cadA* gene was amplified by p1-fw (5'-CTCTGGATCCATGACCAAGCAGTCCGC AGA-3') and p2-rv (5'-CTCTCTGCAGTTACACCA GTGGGGACTTCA-3') oligonucleotides using the plasmid pMA-*cadA*$_{opt}$ as a template. The PCR product was cut with BamHI and PstI, and cloned into the pCH shuttle vector, which was digested with the same restriction enzymes and yielding pCH-*cadA*$_{opt}$. In a similar way, in order to construct the expression plasmids pCH-*cad*$_{opt}$*acnB*$_{opt}$ and pCH-*acnB*$_{opt}$*cad*$_{opt}$, the codon-optimized fusion genes *cadAacnB* were amplified using p1-fw (5'-CTCTGGATCCATGAC CAAGCAGTCCGCAGA-3') and p3-rv (5'-AAAACT GCAGTTACTTGGAGGAGGCTGCCAT-3') oligonucleotides, while the *acnBcadA* fusion genes were amplified with p4-fw (5'-AAAAGGATCCATGGAACTGACCGT GACCGAA-3') and p5-rv (5'-CTCTCTGCAGTTACA CGAGTGGGGACTTCA-3'). Then, the resultant PCR products were digested with BamHI and PstI and cloned into pCH vector cut with the same enzymes resulting in pCH-*cadA*$_{opt}$*acnB*$_{opt}$ and pCH-*acnB*$_{opt}$*cadA*$_{opt}$ plasmids.

2.3. Cultural media and conditions

The bacterial strain was preserved as 25% (v/v) glycerol stocks at –80ºC. For all IA production experiments, BHI

was mainly used for the first preculture where 2 mL of BHI liquid media with 4% (w/v) glucose was inoculated with a single colony of the desired *C. glutamicum* strain from a freshly streaked agar plate and incubated on a rotary shaker at 180 rpm for 8 h at 30ºC. For both the second preculture and the main culture, modified CGXII (mCGXII) medium (1 g/L urea, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 42 g/L 3-morpholinopropanesulfonic acid, 0.25 g/L $MgSO_4$.$7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4.7H_2O$, 0.1 mg/L $MnSO_4$ H_2O, 1 mg/L $ZnSO_4.7H_2O$, 0.2 mg/L $CuSO_4$ $5H_2O$, 20 mg/L $NiCl_2$. $6H_2O$, 0.2 mg/L biotin) with 4% (w/v) glucose as the carbon and energy source was supplemented with a limited amount of nitrogen as described previously [29] was used. The cells of the first preculture were used to inoculate 10 mL of the second preculture in a 50 mL test tube to a final optical density at 600 nm (OD_{600}) of approximately 0.2. After approximately 14 h of incubation at 30ºC and 180 rpm the cells were then used to inoculate the main culture of 20 mL mCGXII in a 100 mL baffled flask to an OD_{600} of approximately 0.2. Additionally, for investigation of different carbon sources for the production of IA, the same mCGXII media was used. Instead of glucose, however, different carbon sources (fructose, sucrose, maltose, mannose and sodium acetate) were tested with a final concentration of 4% (w/v). Moreover, mCGXII was also used with different concentrations of sodium acetate (50, 60, 70, 80, 90, and 100 g/L) as an alternative to glucose as the sole carbon source for the production of IA. Similarly, different concentrations of sodium acetate were combined with different concentrations of sugars like glucose, fructose, sucrose, and maltose, and were tested as a replacement for glucose as the main carbon sources.

2.4. Experimental design

Response surface methodology (RSM) based on central composite design (CCD) was used for optimization of the concentration of the IA through the production process from *C. glutamicum* using two carbon sources as variables (maltose and acetate). A full factorial design with five levels for each variable, five center points and four-star points that resulted in a total of 13 runs was used to optimize the factors for IA production in a shake flask culture. Two coded variables were studied: high (coded value: +1.4142) and low (coded value: −1.4142). The real concentrations of variables were assessed with five levels for each: maltose concentrations (55.85 to 84.14 g/L) and sodium acetate concentrations (57.62 to 72.07). The coded and actual levels of the evaluated parameters and experiment details are shown in Table S1 and S2.

2.5. Flux balance analysis (FBA)

A genome-scale metabolic model of *C. glutamicum*, iCW773, was used in this study [33]. To reveal the IA-biomass solution space, the maximum and minimum fluxes of IA production were calculated, using an objective function as the maximization or minimization of IA production, with each fixed growth rate from zero to the maximum value [34]. All calculations were performed using MATLAB R2021a with COBRA Toolbox v3.0 [35,36] and the opensource GLPK software (http://glpkmex.sourceforge.net/), which is an application that solves linear programming. In this simulation, uptake rates of glucose, maltose, and sodium acetate were set to 0.3 mmol g^{-1} h^{-1}, 0.15 mmol g^{-1} h^{-1}, and 0.9 mmol g^{-1} h^{-1}, respectively, to ensure the same amount of carbon. In case of combined carbon sources (maltose with sodium acetate), uptake rates were set to 0.075 mmol g^{-1} h^{-1} for maltose and 0.45 mmol g^{-1} h^{-1} for sodium acetate, to ensure the same amount of carbon.

2.6. Statistical analysis

Each data is indicated by the average value with standard deviation as an error bar, which were calculated from three biological replicate experiments. Data differences were compared using the paired Student's *t*-test with independent triplicated data. A p value of < 0.05 was considered to be statistically significant. CCD design and analysis of variance (ANOVA) were conducted using the Design Expert 13.0 statistical package (Stat-Ease, Inc.).

2.7. Analysis of organic acids and carbon sources

High-performance liquid chromatography (HPLC) was used to quantify organic acids in the supernatants of the culture. Cultured broth samples were collected and centrifuged (15,000 rpm for 10 min at 4°C). The concentration of IA in the resultant supernatant was measured via HPLC using a diode array detector SPD-20AV at UV210 nm (Shimadzu) equipped with a column (InertSustain 5 um, 150 mm × 4.6 mm internal diameter; GL Science). A mobile phase buffer of 50 mM NaH_2PO_4 with a pH of 2.1 and flow rate of 1.0 mL/min was used, and the temperature of the column oven was adjusted to 40°C. The concentration of sodium acetate in the culture supernatant was also analyzed and measured using an HPLC fitted with a refractive index detector (RID-10A; Shimadzu). An ICSepICE-COREGEL-87H column (300 mm × 7.8 mm of internal diameter; Transgenomic Inc.) was used, and 5 mM H_2SO_4 buffer was used for elution at a flow rate of 0.6 mL/min at 80ºC for 40 min. To evaluate the concentrations of sugars such as glucose, fructose, sucrose, mannose and maltose, a Shim-Pack SPR-Pb column (250 mm × 7.8 mm of internal diameter; Shimadzu) was used at a flow rate of 0.6 mL/min, with MilliQ serving as the mobile phase at 80ºC for 40 min for each sample.

3. Results and Discussion

3.1. IA production by engineered *C. glutamicum* strain

C. glutamicum is widely used to produce a variety of amino acids in the industrial sector. Recently, it has also been used to produce a variety of organic acids [37]. In our experiment, we investigated the ability of the engineered *C. glutamicum* strain to produce IA.

There are only two enzymes involved in the IA production pathway [38]. The first key enzyme is aconitase (*acnB*), which is found in the tricarboxylic acid cycle for the conversion of citric acid to *cis*-aconitic acid, then *cis*-aconitic acid is decarboxylated to IA by the *cadA* gene. In current study these two genes which are involved in IA production pathway were fused to enhance IA production. Three sets of genes were expressed by wild-type *C. glutamicum* ATCC 13032 using the expression vector pCH (with HCE-high constitutive promoter). These genes are *cadA* derived from *A. terreus* with a codon-optimized sequence, and two fused sets of genes, *cadAacnB* and *acnBcadA*, with codon optimized sequences giving the three plasmids pCH-$cadA_{opt}$, pCH-$cadA_{opt}acnB_{opt}$, and pCH-$acnB_{opt}cadA_{opt}$. These plasmids were transformed to wild-type *C. glutamicum* ATCC13032 giving the three strains of *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$, *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}acnB_{opt}$, and *C. glutamicum* ATCC 13032 pCH-$acnB_{opt}cadA_{opt}$.

The expression of the codon-optimized *cadA* and the two sets of fused genes, *cadAacnB* and *acnBcadA*, in the wild-type *C. glutamicum* ATCC 13032 led to IA production in the media with final titers of 0.242, 0.028, and 0.073 g/L, respectively (Fig. S1A). The glucose was completely consumed by all three constructed strains, and cell growth was the same for all of them (Fig. S1B and S1C). Consequently, it is obvious that the recombinant strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was better than the two other constucted strains with the fused genes in the production of IA with a final titer of 0.242 g/L IA (Fig. S1A), and the fusion between the *cadA* gene from *A. terreus* and the *acnB* gene from *C. glutamicum* and their expression in *C. glutamicum* did not increase the concentration of IA in the media, while the expression of *cadA* gene only resulted in a better final concentration. Therefore, we suggest that the fusion strategy was not an appropriate strategy for expression of both genes and enhancement of IA production in *C. glutamicum*. Similarly, in a previous study using *E. coli* for the production of IA, fused *acnB* and *cadA* could not be expressed in soluble form. That result showed that the fusion strategy was not suitable for expressing the two fused enzymes in *E. coli*, which resulted in low levels of IA (0.117 g/L) after 48 h of fermentation [39]. The self-assembly of *acnB* and *cadA*, however, improved the IA titer to 0.22 g/L, which was still low and not significant. In a similar manner, our results showed that the fusion between the two genes *acnB* and *cadA* was not suitable for the production of IA from *C. glutamicum* giving low IA concentrations similar to that obtained by *E. coli*.

3.2. Comparison of different strains of *C. glutamicum* for production of IA

The present study is the first to evaluate the use of different strains of *C. glutamicum* to express the *cadA* gene for IA production. The plasmid pCH-$cadA_{opt}$ was chosen for transformation to an additional three strains of *C. glutamicum*: ATCC 21799, ATCC 21253, and ATCC 13287 to examine the expression capability of *cadA* gene in other *C. glutamicum* wild-strains. This resulted in the following three strains: *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$, *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$, and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$. These three strains were evaluated for production of IA from glucose under nitrogen-limited conditions. The results revealed that best strain forIA production was *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ with 0.242 g/L IA followed by *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$ with 0.15 g/L IA, *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$ with 0.045 g/L IA, and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$ with 0.02 g/L (Fig. S2A).

Additionally, the cell growth of *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ proved to be better than the other strains (Fig. S2C). The glucose was completely consumed only by *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. With the other strains, 3.53, 4.60, and 3.60 g/L glucose was not consumed by *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$, *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$, and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$, respectively (Fig. S2B). Therefore, the wild-type *C. glutamicum* ATCC 13032 of *C. glutamicum* gave the highest IA production titer, and cell growth with complete glucose consumption. Using other strains resulted in lower IA production titer, which could be due to low *cadA* gene expression, slow growth and incomplete consumption of glucose. For this reason, *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was chosen for further investigation.

3.3. Evaluation of different carbon sources for production of IA in *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$

There are various carbon sources that *C. glutamicum* uses for growth and energy production including; monosaccharides like glucose, fructose, and mannose; disaccharides like sucrose and maltose; and, organic acids like acetic acid [8]. In previous studies, IA was produced from metabolically engineered *C. glutamicum* ATCC 13032 with a final titer of 7.8 g/L from glucose [29] and 29.2 g/L from sodium acetate [28]. Compared with previous studies, our study is

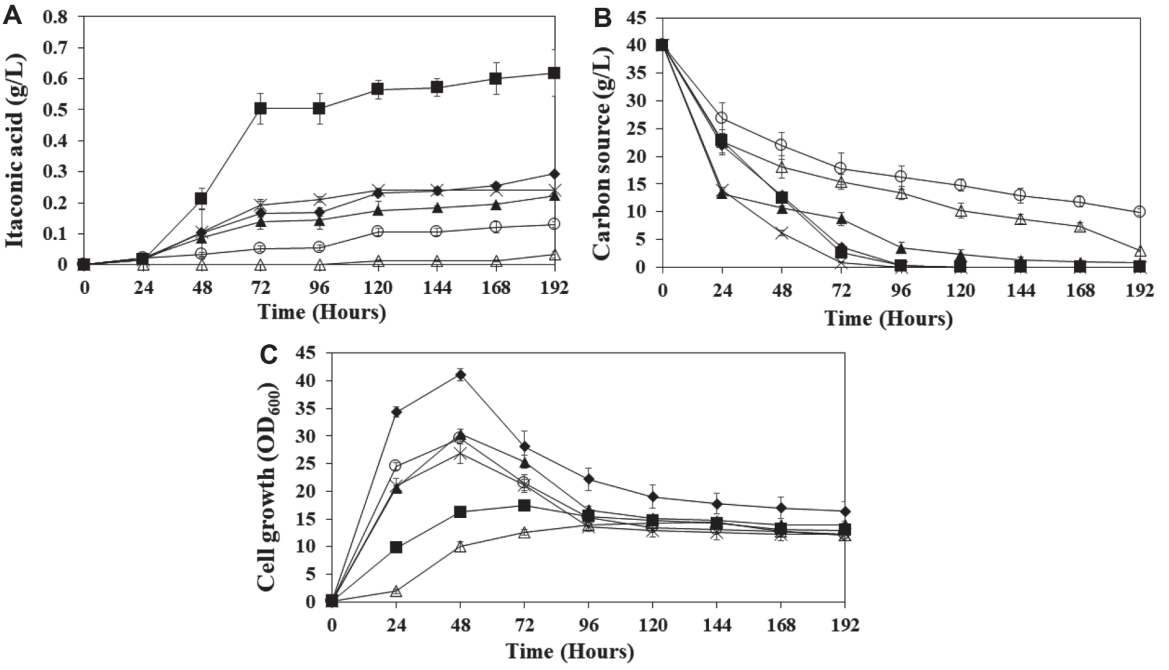

Fig. 1. Itaconic acid (IA) production by *Corynebacterium glutamicum* ATCC 13032 pCH-$cadA_{opt}$ from different carbon sources. IA production (A), carbon source consumption (B), and cell growth (OD_{600}) (C) are indicated. *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different carbon sources including glucose (multiplication symbols), fructose (closed triangles), sucrose (open circles), maltose (closed diamonds), mannose (open triangles), and sodium acetate (closed squares) to obtain a final carbon source concentration of 40 g/L, under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

the first to compare between different carbon sources for the production of IA from *C. glutamicum*. Six carbon sources (glucose, fructose, sucrose, maltose, mannose, and sodium acetate) were evaluated for production of IA from the selected strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ under nitrogen-limited conditions to improve the substrate availability for IA production. The results revealed that the best carbon source for IA production was sodium acetate with a final IA titer of 0.62 g/L, and the lowest carbon source was mannose with a final IA titer of 0.033 g/L (Fig. 1A). The IA titers from maltose, glucose, fructose, and sucrose were 0.293, 0.242, 0.223, and 0.13 g/L, respectively (Fig. 1A).

It was found that sodium acetate, glucose, and maltose were completely consumed in the media, while fructose, sucrose, and mannose were not (Fig. 1B). Moreover, the best cell growth was reached when using maltose as a carbon source while the lowest was with mannose followed by sodium acetate (Fig. 1C).

3.4. Usage of different sodium acetate concentrations for IA production

In a previous study, sodium acetate with different concentrations of 1, 5, 10, 15, 20, 25, and 30 g/L were used to determine its effect on bacterial growth. *C. glutamicum* was effective in growing on all concentrations with the full consumption of sodium acetate [40], and has progressively shown that it was capable of growing on sodium acetate as the main carbon source at substrate concentration as high as 60 g/L.

In the current study, six different concentrations of sodium acetate (50, 60, 70, 80, 90, and 100 g/L) were investigated for IA production in *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ under nitrogen-limited conditions, resulting in final IA titer of 1.81, 2.79, 3.55, 2.74, 2.69, and 1.45 g/L using 50, 60, 70 ,80, 90, and 100 g/L of sodium acetate, respectively. By increasing sodium acetate concentration in the media, the final concentration of IA was improved (Fig. 2A), and the highest IA titer reached was 3.55 g/L using 70 g/L sodium acetate. However, the IA concentration began to decrease at concentration of sodium acetate higher than 70 g/L, cell growth was also decreased with the increase in the sodium acetate concentration in the media (Fig. 2C), showing that increasing the concentration of sodium acetate inhibited cell growth. In addition, *C. glutamicum* couldn't consume all the sodium acetate that was present in the media after 60 g/L (Fig. 2B). Consequently, evaluation of different concentrations of sodium acetate showed that increasing the sodium acetate concentration could increase the production of IA in the medium. Despite the increase

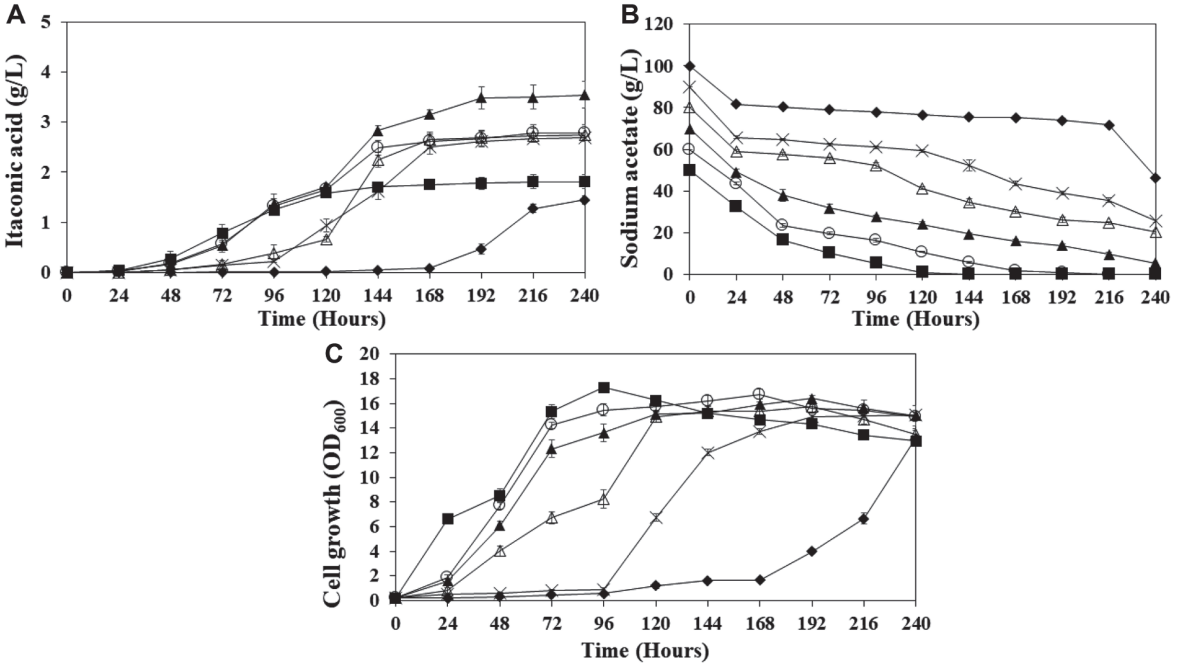

Fig. 2. Dose effect of sodium acetate on itaconic acid (IA) production. IA production (A), sodium acetate consumption (B) and cell growth (OD_{600}) (C) were indicated. *Corynebacterium. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media with different concentrations of sodium acetate: 50 g/L (closed squares), 60 g/L (open circles), 70 g/L (closed triangles), 80 g/L (open triangles), 90 g/L (multiplication symbols) and 100 g/L (closed diamonds) as the main carbon source under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

in the final IA titer, increasing the sodium acetate in the medium to a concentration higher than 70 g/L inhibited cell growth (Fig. 2C), and the sodium acetate was not completely consumed in the medium (Fig. 2B), which resulted in decrease in the final IA titer (Fig. 2A). Similarly, in previous study, it has been shown that concentrations of sodium acetate in the media higher than 50 g/L did not lead to an obvious rise in biomass, although the nitrogen source that existed in the medium should help in increasing the cell density [41]. It was found that there was an obvious expanding in the lag phase by increasing the concentration of sodium acetate to more than 10 g/L. This effect could have been a result of the specific inhibitory effects of sodium acetate such as an uncoupling of the trans-membrane pH gradient [42], and that could possibly influence the growth behavior once the crucial concentration is reached.

3.5. Combination between sodium acetate and different sugars for improvement of IA production

Most microorganisms can use more than one substrate for their growth and for the production of different metabolites and adapt their metabolism to the presence of certain substrates [1]. Previous studies have shown that *C. glutamicum* has the ability to simultaneously use more than one carbon source [10,12]. Therefore, depending on this concept, mixtures of sodium acetate and other sugars were evaluated for enhancing cell growth and IA production in *C. glutamicum*.

In this study, it was found that sodium acetate is the best carbon source for the production of IA with a final IA titer of 0.62 g/L (Fig. 1A), however, sodium acetate resulted in a low cell growth compared to other sugars like glucose, fructose, sucrose and maltose (Fig. 1C). Consequently, the combination of sugars like glucose, fructose, sucrose and maltose with sodium acetate was evaluated with different three mixture concentrations: (20 + 20, 20 + 40 and 40 + 40) g/L respectively of glucose, fructose, sucrose and maltose with sodium acetate. The results showed that maltose-sodium acetate mixture was the best combination for production of IA with final IA titer of 4.96 g/L and a molar yield 0.2 mol/mol from a mixture of (40 + 40) g/L of maltose and sodium acetate (Fig. 3D), followed by glucose and fructose mixtures with sodium acetate with final IA titer of 3.43 g/L with a molar yield of 0.097 mol/mol from a mixture of (40 + 40) g/L of glucose and sodium acetate (Fig. 3A), and 2.17 g/L with a molar yield of 0.055 mol/mol from a mixture of (40 + 40) g/L of fructose and sodium acetate (Fig. 3B), while the lowest IA titer was from sucrose-sodium acetate mixture with final IA titer of 0.76 g/L and a molar yield of 0.032 mol/mol from a mixture of (40 + 40)

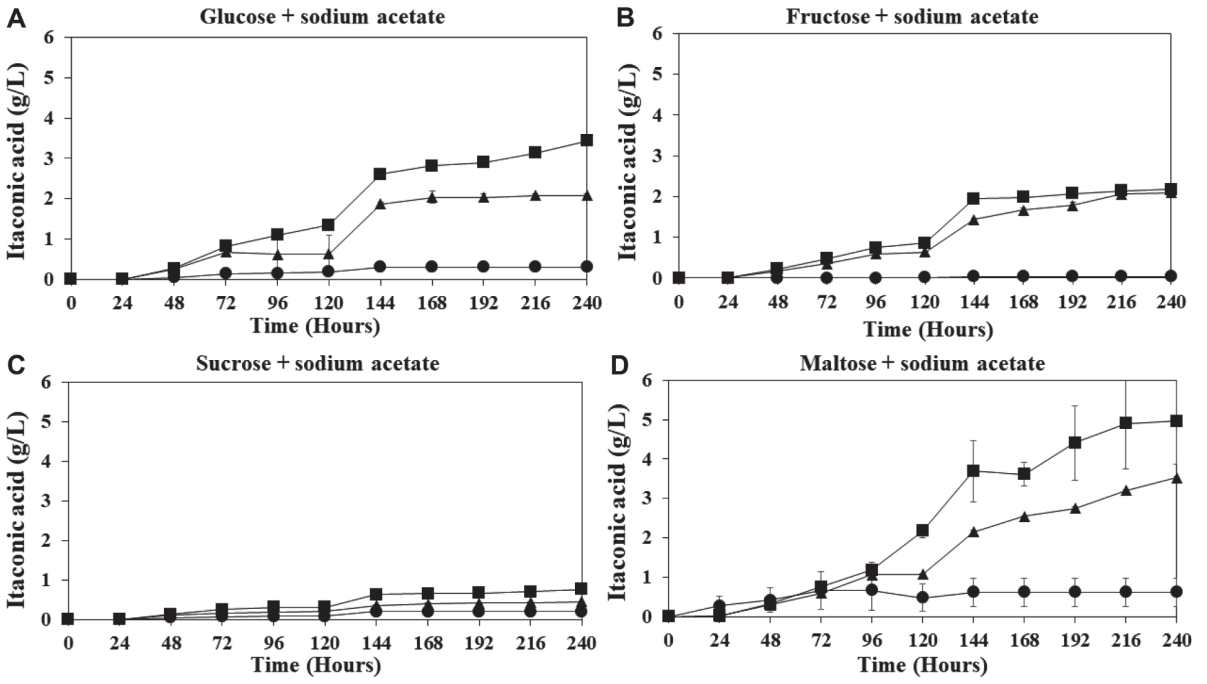

Fig. 3. The effect of combinations of sodium acetate and different sugars on itaconic acid (IA) production in *Corynebacterium glutamicum* ATCC 13032 pCH-$cadA_{opt}$ strain. IA production was indicated where *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing glucose and sodium acetate (A), fructose and sodium acetate (B), sucrose and sodium acetate (C), and maltose and sodium acetate (D) as main carbon sources and under nitrogen-limited conditions. Different combinations of glucose, fructose, sucrose and maltose with sodium acetate: 20 + 20 g/L (closed circles), 20 + 40 g/L (closed triangles), and 40 + 40 g/L (closed squares), respectively, were checked. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

g/L of sucrose and sodium acetate (Fig. 3C).

Furthermore, the cell growth in the case of maltose-sodium acetate mixture was shown to be the highest compared to other combinations (Fig. 4D). It is also better than cell growth in the case of using sodium acetate only as a main carbon source, even after increasing sodium acetate concentration in the medium (Fig. 1C and 2C). Moreover, in the case of other sugars combinations with sodium acetate, the cell growth was not high compared to using sodium acetate only as a sole carbon source (Fig. 4A-4C). Consequently, it was found that maltose-sodium acetate mixture resulted in the highest final IA titer and cell growth.

Additionally, In case of combination between glucose with sodium acetate, only by using (20 + 20) g/L combination, both sodium acetate and glucose were consumed. In case of higher concentrations, the sodium acetate was consumed faster, and glucose was not totally consumed (Fig. 5). In the case of combination between fructose and sodium acetate, both fructose and sodium acetate were completely consumed (Fig. 6). Similar to glucose and sodium acetate mixture, only by using (20 + 20) g/L combination, both sucrose and sodium acetate were consumed (Fig. 7). Both maltose and sodium acetate were completely consumed in the case of all concentrations (Fig. 8). However, the differences in consumption of carbon sources in different mixtures, all sugars combinations with sodium acetate were co-utilized by *C. glutamicum* and supported monophasic growth.

3.6. CCD experiment for the production of IA using a combination of maltose and sodium acetate

In this study, the highest IA production titer was achieved by utilizing sodium acetate as a carbon source by *C. glutamicum* (Fig. 1A) while maltose recorded the highest cell growth (Fig. 1C). The combination between maltose and sodium acetate resulted in the highest IA titer and the highest cell growth (Fig. 3 and 4). As a consequence of these results, RSM (Fig. S3) using CCD was used to study the effect that combining maltose and sodium acetate with different concentrations (Table S1 and S2) would exert on IA production in *C. glutamicum.* A quadratic model was applied using Design Expert 13 software according to the coded values of the quadratic equation:

$$Itaconic\ acid = +9.03 - 2.22A + 0.3676B - 0.09AB - 0.2737A^2 - 1.94B^2$$

Furthermore, a Fisher test (F-test) was applied for evaluation of the statistical importance of the extracted

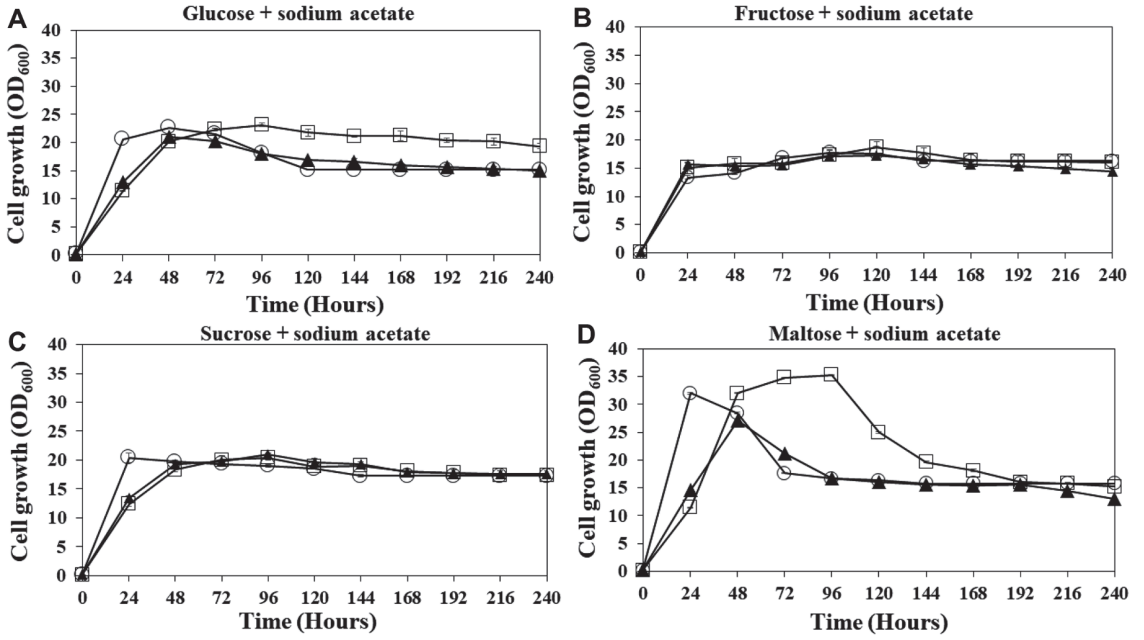

Fig. 4. The effect of combinations of sodium acetate and different sugars on cell growth by *Corynebacterium glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain. Cell growth (OD_{600}) was indicated, where *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing glucose and sodium acetate (A), fructose and sodium acetate (B), sucrose and sodium acetate (C) and maltose and sodium acetate (D) as main carbon sources and under nitrogen-limited conditions. Different combinations of glucose, fructose, sucrose and maltose with sodium acetate: 20 + 20 g/L (open circles), 20 + 40 g/L (closed triangles), and 40 + 40 g/L (open squares), respectively, were checked. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

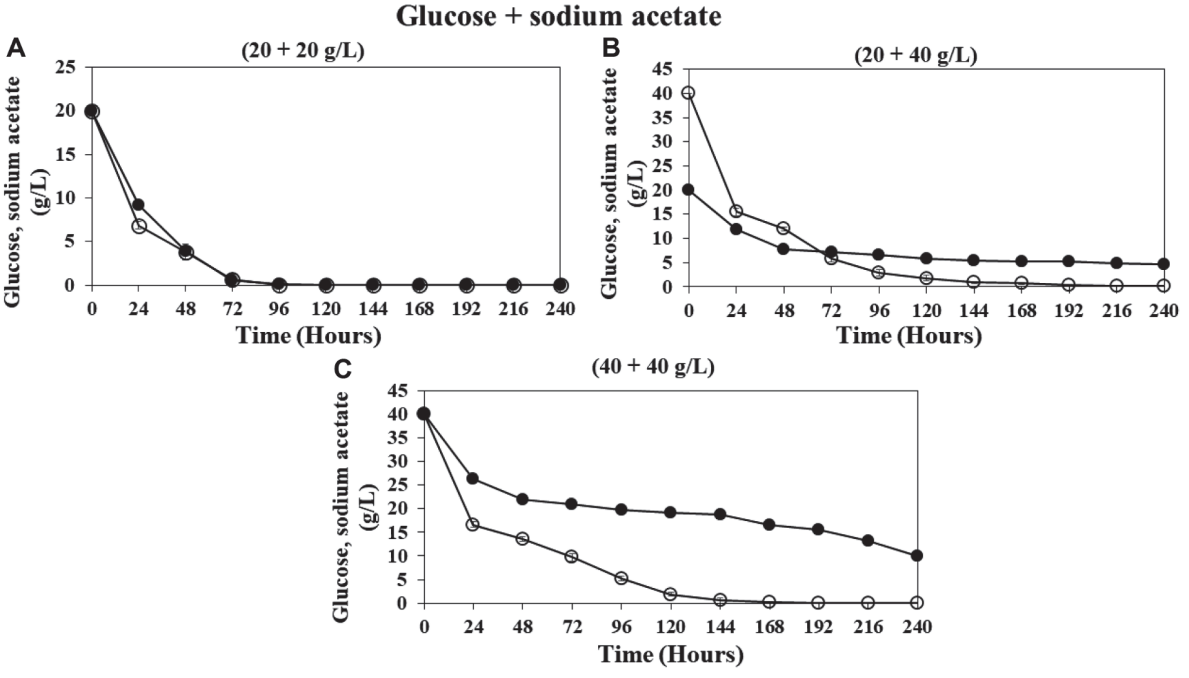

Fig. 5. Time course consumption of combined glucose and sodium acetate by *Corynebacterium glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$. Glucose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of glucose and sodium acetate: 20 + 20 g/L (A), 20 + 40 g/L (B) and 40 + 40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

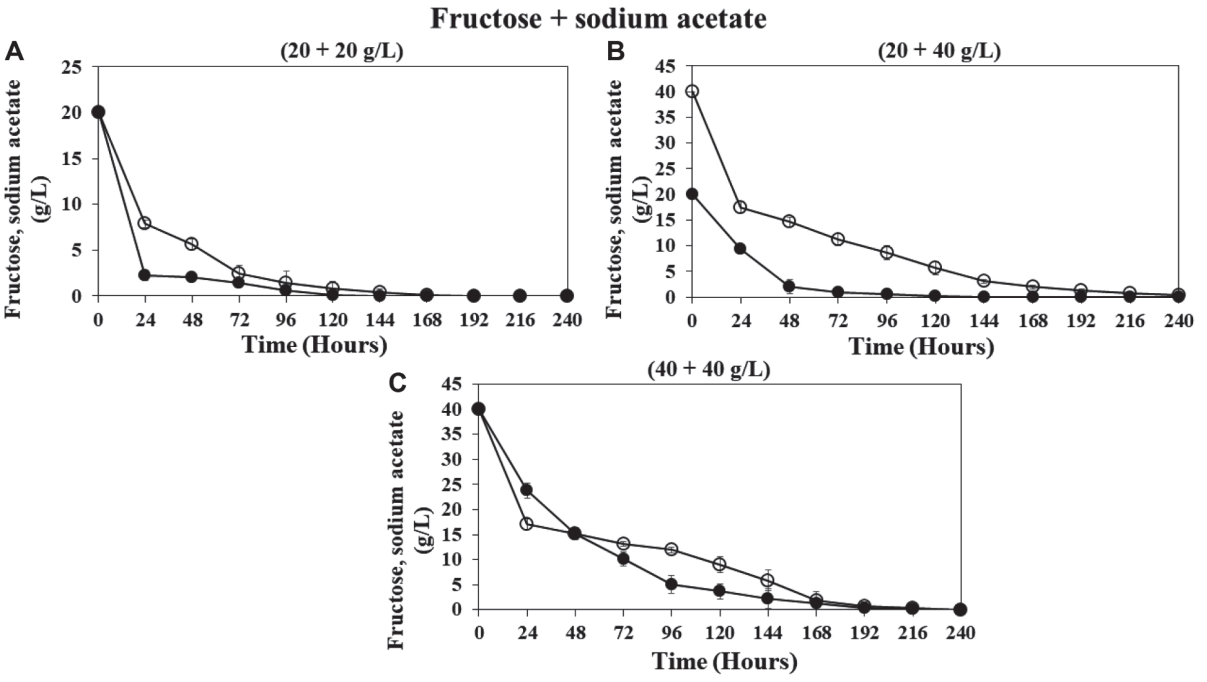

Fig. 6. Time course consumption of combined fructose and sodium acetate by *Corynebacterium glutamicum* ATCC 13032 pCH-$cadA_{opt}$. Fructose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of fructose and sodium acetate: 20 + 20 g/L (A), 20 + 40 g/L (B) and 40 + 40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

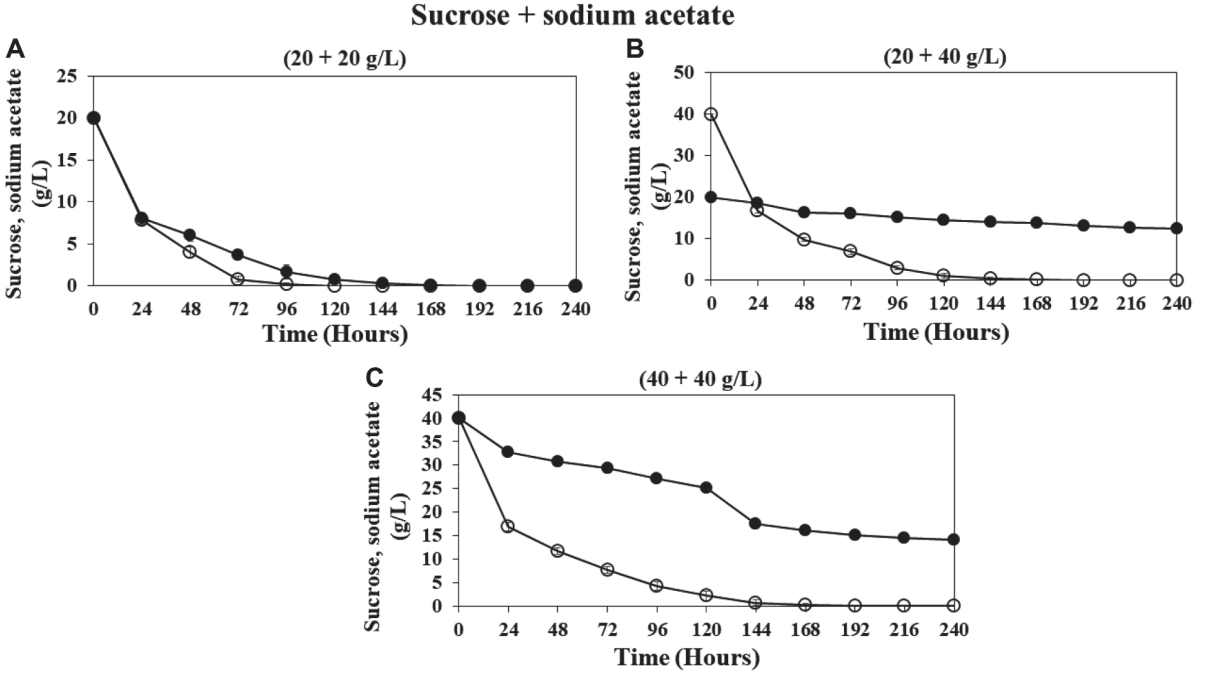

Fig. 7. Time course consumption of combined sucrose and sodium acetate by *Corynebacterium glutamicum* ATCC 13032 pCH-$cadA_{opt}$. Sucrose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of sucrose and sodium acetate: 20 + 20 g/L (A), 20 + 40 g/L (B) and 40 + 40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

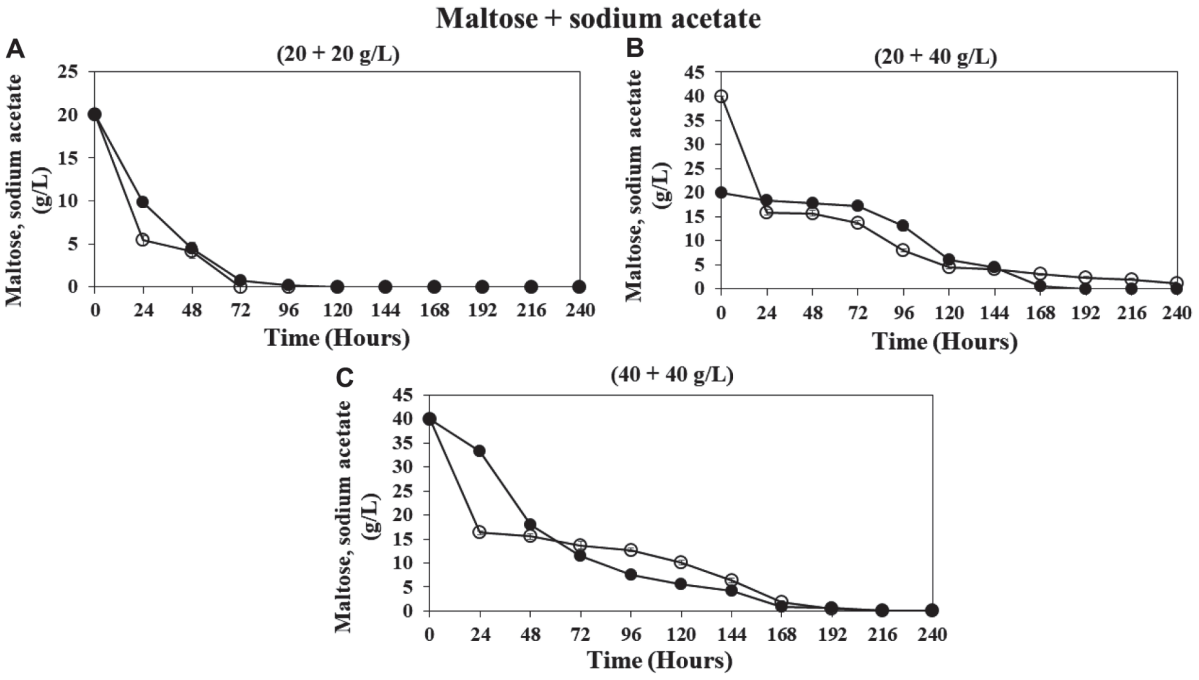

Fig. 8. Time course consumption of combined maltose and sodium acetate by *Corynebacterium glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$. Maltose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of maltose and sodium acetate: 20 + 20 g/L (A), 20 + 40 g/L (B) and 40 + 40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

model, where the ANOVA was calculated by dividing the mean squares by the model residual mean square (Table S3). The ANOVA results emphasized the possibility of applying the model. The resultant F-value for IA (17.92), and the achieved probability (p value = 0.0007) with a low value confirmed also the model significance. For IA, the A and B^2 terms were found to be significant while the B, AB and A^2 terms did not have a significant effect in the design space of the model. Consequently, only the terms A and B^2 were kept.

A lack-of-fit test was applied to compare the residual error with the pure error. The p value for the lack-of-fit test showed a value greater than 0.05, as shown in Table S3, which reflects the insignificance of the lack-of-fit and reflected the model acceptance. Calculation of the IA equation for adequate precision or signal-to-noise value gave a value of 12.4506, which is higher than 4 and indicates the accuracy and significance of the model. In addition, the agreement between the predicted values and actual values was confirmed by using the predicted R^2 value.

3.7. Optimization of IA production parameters

Different combinations of maltose and acetate were examined to reach optimum concentrations. When combined, concentrations of 55.85 and 65 g/L of maltose and sodium acetate, respectively, resulted in the highest IA titer of 12.63 g/L (Fig. 9). Additionally, these results confirmed the nature of the monophasic growth of *C. glutamicum* where both acetate

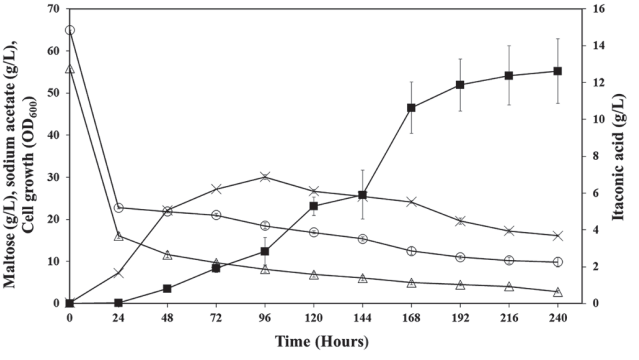

Fig. 9. Time course profile of itaconic acid (IA) production by the optimized mixture of maltose and sodium acetate. IA production (closed squares), maltose consumption (open triangles), sodium acetate consumption (open circles) and cell growth (OD_{600}) (multiplication symbols) were charted for the strain *Corynebacterium glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain was inoculated at an initial OD_{600} of 0.2 in mCGXII media containing maltose and sodium acetate with final concentrations of 55.85 and 65 g/L, respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

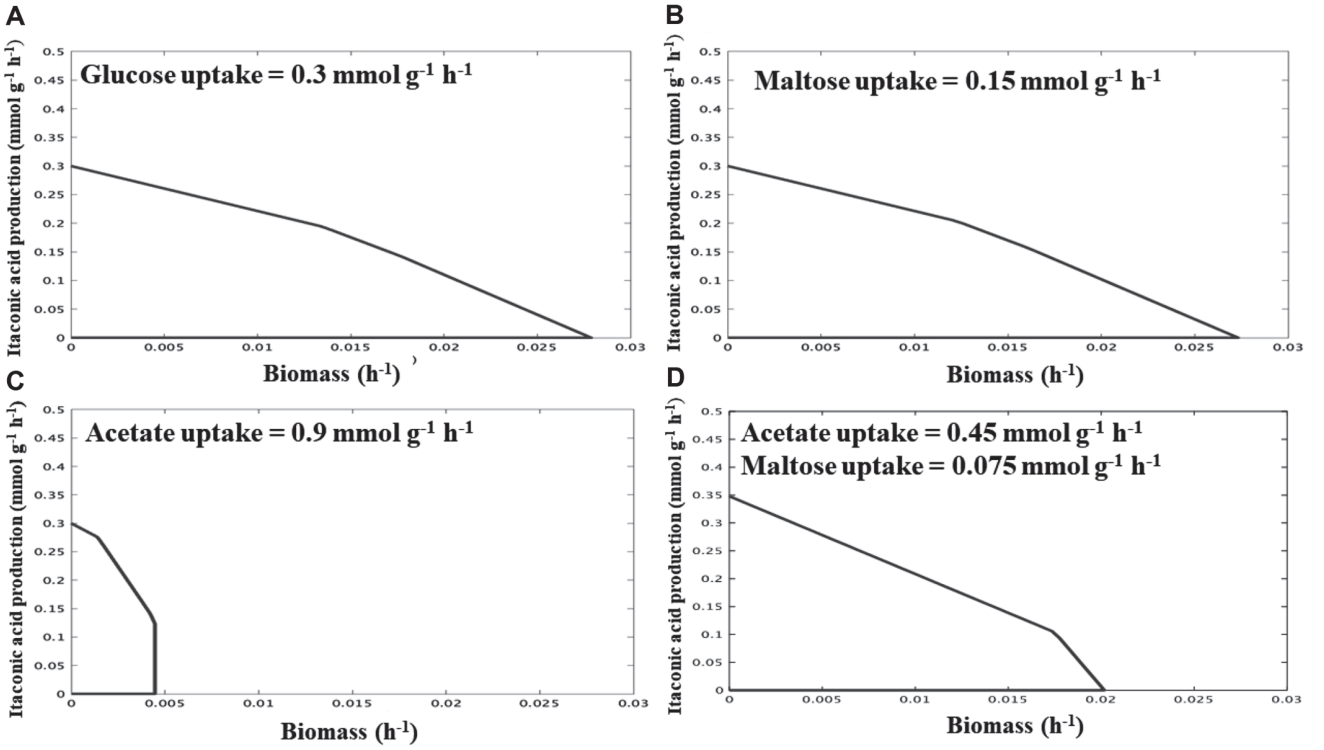

Fig. 10. Flux balance analysis. (A) Itaconic acid (IA) production in case of using glucose as a carbon source where glucose uptake was set to 0.3 mmol g^{-1} h^{-1}. (B) IA production in case of using maltose as a carbon source where glucose uptake was set to 0.15 mmol g^{-1} h^{-1}. (C) IA production in case of using sodium acetate as a carbon source where sodium acetate uptake was set to 0.9 mmol g^{-1} h^{-1}. (D) IA production in case of co-utilizing of maltose and sodium acetate as main carbon sources where maltose uptake was set to 0.075 mmol g^{-1} h^{-1} and acetate uptake was set to 0.45 mmol g^{-1} h^{-1}.

and maltose were simultaneously consumed, and cell growth was enhanced. Also, the reduction in the lag phase period is another improvement over using sodium acetate only that was characterized by a longer lag phase, particularly when using higher concentrations (Fig. 2C). Therefore, using CCD for optimization of IA production using maltose and sodium acetate as main carbon sources showed an enhancement of cell growth and IA production giving final IA titer of 12.63 g/L (Fig. 9) with a molar yield of 0.38 mol/mol, where *C. glutamicum* was able to simultaneously consume both carbon sources. Similarly, the effect of maltose on glucose and sodium acetate usage by *C. glutamicum* has also been investigated previously for improvement of L-valine productivity [9] where maltose was co-metabolized with both glucose and sodium acetate by *C. glutamicum*. Moreover, an enhancement in the productivity of L-valine has been investigated by co-metabolizing of acetate and maltose by *C. glutamicum*. Adding maltose also during the growth on a combination of glucose and sodium acetate has resulted in effective glucose consumption, as well as in improvement in L-valine productivity. The results of these previous studies agree with our results.

3.8. FBA

The FBA was calculated to reveal the IA-biomass solution space, and the maximum and minimum fluxes of IA production were calculated. To equalize the number of carbon, uptake rates of glucose, maltose, and sodium acetate were set to 0.3 mmol g^{-1} h^{-1}, 0.15 mmol g^{-1} h^{-1}, and 0.9 mmol g^{-1} h^{-1}, respectively. In case of maltose with sodium acetate, uptake rates were set to 0.075 mmol g^{-1} h^{-1} for maltose and 0.45 mmol g^{-1} h^{-1} for sodium acetate, and O_2 uptake was set to 0.8 mmol g^{-1} h^{-1} which is a bit more anaerobic than the optimal value. FBA results also revealed that the biomass and IA production in the case of co-feeding of maltose and sodium acetate is better than sodium acetate (Fig. 10C and 10D). Consequently, FBA also supported our results including biomass and IA production, and it proved that the IA production could be better when using sodium acetate and maltose as a combined carbon sources than when maltose and sodium acetate were separately used. The tendency of cell growth also agreed with the results by FBA (Fig. 1 and 10).

4. Conclusion

In this study, using maltose with sodium acetate as a combined carbon source mixture for the production of IA in *C. glutamicum* enhanced the cell growth, and decreased the lag phase giving a final IA titer of 12.63 g/L. This is the first study that has evaluated the effect of combined substrate mixtures on the production of IA, and the results support the possibility of a simultaneous utilization of different carbon sources for IA production by *C. glutamicum*.

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Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-023-0091-7) contains supplementary material, which is available to authorized users.

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